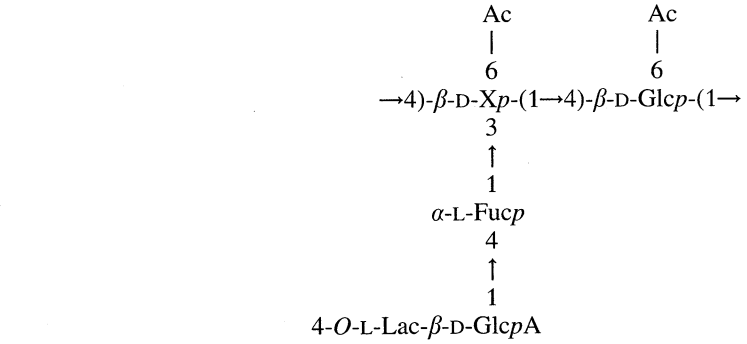
Structural investigation of the exopolysaccharide produced by *Pseudomonas flavescens* strain B62

Degradation by a fungal cellulase and isolation of the oligosaccharide repeating unit

Pseudomonas flavescens strain B62 (NCPPB 3063) is a recently described bacterium isolated from walnut blight cankers. This strain has been designated as the type strain of a *Pseudomonas* rRNA group-I species. Strain B62 produced a mixture of two exopolysaccharides, differing in weight average relative molecular mass and composition. Only the most abundant exopolysaccharide (90% by mass), corresponding to the one with the lower molecular mass, was investigated by use of methylation analysis, partial acid hydrolysis, and NMR spectroscopy. The polysaccharide was depolymerised by the action of the cellulase produced by *Penicillum funiculosum* and the oligosaccharide obtained, corresponding to the repeating unit, was characterised by NMR spectroscopy and ion-spray mass spectrometry. The repeating unit of the B62 exopolysaccharide is



where X is glucose (75%) or mannose (25%), and Lac is lactate. The *O*-acetyl groups are present only on 75% of the repeating units, and they are linked to the C6 of the hexose residues in non-stoichiometric amounts.

Keywords: Pseudomonas; exopolysaccharide; structure; cellulase; NMR.

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Abbreviations. EPS, exopolysaccharide; dHex, deoxyhexose; Glc*p*A, glucuronic acid; Hex, hexose; HexA, hexuronic acid; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; Lac, lactate; M_n, number average molecular mass; M_w, weight average relative molecular mass.

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Ion-spray mass spectrometry. The mass spectra were recorded on a API-I PE SCIEX quadrupole mass spectrometer

Table 2. ^1H and ^{13}C chemical shifts of 4-*O*-L-Lac-β-D-Glc*p*A-(1→4)-L-Fuc. The chemical shifts are given relative to internal acetone set to 2.225 ppm and 31.07 ppm for ^1H and ^{13}C, respectively.

Residue	Nucleus	Proton or carbon					
		1	2	3	4	5	6
		ppm					
-4)GlcA(β1-	H	4.49	3.48	3.65	3.37	3.77	
	C	103.5	74.3	75.1	81.9	76.7	175.9
-4)Fuc(α1-	H	5.23	3.78	3.78	3.99	4.28	1.29
	C	93.1	69.4	69.5	81.9	67.3	16.2
-4)Fuc(β1-	H	4.59	3.45	3.60	3.93	3.87	1.33
	C	97.0	73.0	73.0	81.0	71.6	16.2
Lac	H	1.33	3.94				
	C	19.7	79.1	182.7			

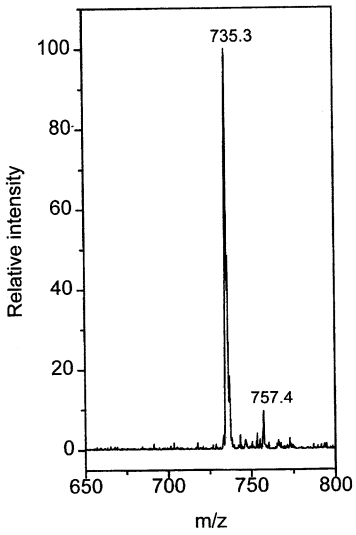

Fig. 1. Negative-mode ion-spray mass spectrum of CL1.

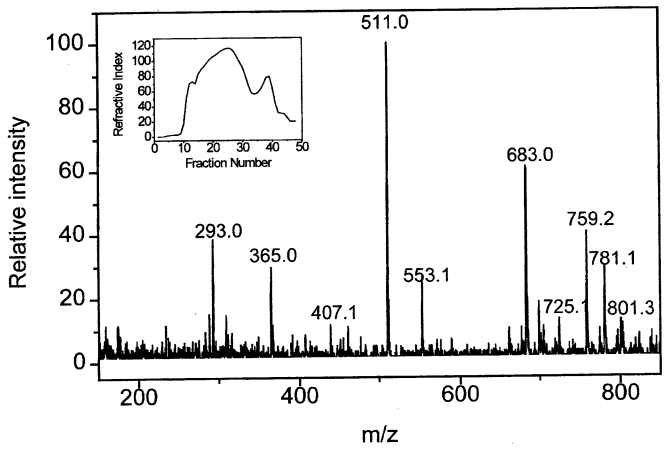

Fig. 2. Positive-mode collision-induced dissociation (orifice voltage 150 V, ion-spray voltage 5000 V) of the mixture of CL1 and CL1-Ac. In the inset the Biogel P2 elution profile of the products obtained upon treatment of B62 EPS with cellulase is shown.

mannose. Moreover, the data reported indicated that CL1 is a mixture of two tetrasaccharides, differing from each other by one sugar residue.

The absolute configuration of the sugar residues was determined on a sample of the CL1 oligosaccharide, which was subjected to methanolysis, treatment with boron tribromide to remove the lactyl substituent, and butanolysis. The configuration was shown to be L for the fucose residues and D for all the other sugars.

Location of the *O*-acetyl substituents. A sample of B62 EPS was treated with cellulase from *P. funiculosum* and purified as described above. The elution profile obtained upon separation of the products on a Biogel P2 column was very different from the one obtained when the deacetylated B62 EPS was used. It showed two main peaks, one at the exclusion volume and one with a retention time corresponding to the tetrasaccharide CL1, both not separated from a very broad band that eluted throughout the range of the inclusion volume of the column (Fig. 2). Positive-ion-mode ion-spray MS of single fractions located the tetrasaccharides corresponding to non acetylated CL1 and acetylated CL1 (CL1-Ac). The fraction containing a high amount of CL1-Ac (fraction 34) was subjected to ion-spray MS analysis in the positive-ion mode, and the tetrasaccharide was fragmented by increasing the orifice voltage to 150 V (Fig. 2). The fragmentation pattern established the sequence Lac-HexA-dHex-Hex-Hex, where Hex is hexose, dHex is deoxyhexose and HexA is hexuronic acid, which is in agreement with the results from methylation analysis, enzymatic degradation and partial acid hydrolysis, and indicated that the *O*-acetyl substituents are located on the hexopyranosyl residues of the backbone (Table 3). Fractions 16–40 were pooled, freeze/dried and methylated under neutral conditions (Prehm, 1980). The results of the GLC/MS analysis of the derived alditol acetates indicated that *O*-acetyl substituents are present on 75% of the repeating units, and that they are linked to C6 of all the mannopyranosyl residues and to part of C6 of the glucopyranosyl residues. These results were corroborated by inspection of the ^{13}C-NMR spectrum of B62 EPS (data not shown).

NMR studies of the oligosaccharide CL1. The ^1H-NMR spectrum of CL1 (Fig. 3) showed resonances attributable to three α (5.31, 5.22 and 5.09 ppm) and four β (4.75, 4.66, 4.52 and 4.49 ppm) anomeric protons, which were named a–g in order of decreasing chemical shift. Integration data indicated that CL1 is a tetrasaccharide, although the area for each of the anomeric signals was less than one, except for resonance g, which resulted after deconvolution of the overlapping signals f and g (Pons et al., 1996). The signals at 5.22 ppm (b) and 4.66 ppm (e) were

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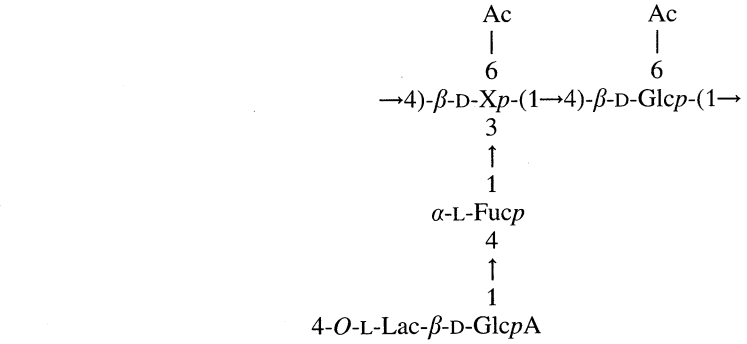

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equipped with an articulated ion spray and connected to a syringe pump for the injection of the samples. The instrument was calibrated using a poly(propylene glycol) mixture [33 μM polypropylene glycol of number average molecular mass (M_n) = 425, 0.1 mM poly(propylene glycol) M_n = 1000, and 0.2 mM poly(propylene glycol) M_n = 2000], 0.1% acetonitrile and 2 mM ammonium formate in 50% aqueous methanol.

The oligosaccharides were dissolved in 50% aqueous acetonitrile. Ammonium acetate at 63 μM and 0.13 mM was used as ionising agent in the positive-ion and negative-ion modes, respectively. The injection flow rate was 5 μl/min. When the analyses were conducted in the positive mode, the ion-spray voltage was 5000 V and the orifice potential was 50 V. In the negative mode, the ion-spray voltage was set at −5000 V and the orifice potential at −50 V. The spectra were recorded using a step size of 0.1 Da. When fragmentation was needed, the orifice potential was set at 150 V.

RESULTS

Composition. EPS was subjected to anion-exchange chromatography on DEAE-Sepharose and eluted as a single peak between 0.2 M and 0.4 M NaCl. However, when gel-filtration chromatography on Sephadex S-400 was performed, the elution profile of the EPS showed two peaks. Moreover, high-performance size-exclusion chromatography indicated that the higher-molecular-mass component, corresponding to about 10% (by mass) had an M_w of 3×10^5 while the lower-molecular-mass polysaccharide had an M_w of 9×10^3. After separation by gel filtration, the two polymers were hydrolysed, their neutral sugars converted to alditol acetates which were then analysed by GLC. The results indicated that the higher-molecular-mass component contained fucose, arabinose, mannose and glucose in the molar ratio 1:1.2:1.3:5, while the lower-molecular-mass component was constituted of fucose, mannose and glucose in the molar ratio 1:0.4:2.5. The composition analysis thus showed that the latter component was not a degradation product of the former. Since the lower-molecular-mass component was the most abundant (90% by mass), it was named B62 EPS, and it was thoroughly investigated, while no further studies were performed on the higher-molecular-mass component. The purification of large amounts of B62 EPS was achieved easily by ultrafiltration on a membrane with a cut-off of 100 kDa. The retentate was subjected to further ultrafiltration on a membrane with a cut-off of 300 kDa, first in water, then in 0.1 M NaCl. The three ultrafiltered solutions contained only B62 EPS, as shown by gel-filtration chromatography on Sephadex S-400, the higher-molecular-mass polymer being in the retentate. The three solutions obtained upon ultrafiltration were pooled, dialysed and lyophylised. The same ultrafiltration procedure was performed on a deacetylated sample of B62 EPS. Further structural studies were performed only on samples of B62 EPS and deacetylated B62 EPS purified by ultrafiltration.

After carboxyl reduction of B62 EPS, hydrolysis, and derivatisation of the neutral sugars to alditol acetates, GLC analysis showed the presence of fucose, mannose, glucose and a slow-eluting compound in the molar ratio 1:0.4:1.7:1.1. The structure of the latter residue was identified as 4-*O*-hydroxyisopropylhexitol acetate by electron-impact GLC-MS analysis (Lindberg et al., 1976). The retention time of this residue (on a SP2330 GLC capillary column) was the same as that of the alditol acetate derived from the carboxyl reduced 4-*O*-L-lactyl-D-glucuronic acid and differed from that one of the D-Lac stereoisomer, thus showing that the slow-eluting component is the reduction product of 4-*O*-L-lactyl-D-glucuronic acid.

Table 1. Methylation analysis of B62 EPS and the oligosaccharide CL1. Retention times are relative to 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-fucitol. Molar ratio values, except those of Glc1,2,3,5,6Me_5-ol and Lac-GlcA, were corrected by use of effective carbon-response factors (Sweet et al., 1975). I, methylated B62 EPS; II, carboxy-reduced, methylated B62 EPS; III, methylated CL1; IV, carboxy-reduced, methylated CL1. R, hydroxyisopropyl (both the primary alcoholic groups are dideuterated according to GLC/MS).

Sugar	Retention time	Molar ratio of			
		I	II	III	IV
Glc1,2,3,5,6Me_5-ol[a]	0.74	0	0	0.64	0.81
Fuc2,3Me_2	1.00	1.00	1.00	1.00	1.00
Glc2,4,6Me_3	1.11	0	0	0.84	0.94
Man2,4,6Me_3	1.12	0	0	0.31	0.26
Glc2,3,6Me_3	1.19	1.14	1.14	0	0
Man2,6Me_2	1.24	0.28	0.19	0	0
Glc2,6Me_2	1.28	0.75	0.62	0	0
Glc2,3$Me_2$4R	1.55	0	1.08	0	1.00

[a] C1 deuterated.

Methylation analysis and related experiments. The results of methylation analysis of the B62 polysaccharide (Table 1), with and without reduction of the methyl ester groups, showed the presence of 4-linked fucopyranosyl, 4-linked glucopyranosyl, 3,4-linked mannopyranosyl, 3,4-linked glucopyranosyl, and 4-*O*-lactyl-glucopyranosiduronic acid. The uneven molar ratios of the branched residues, and that their total sum is one, suggest that the repeating unit is a tetrasaccharide with either a 3,4-linked glucose or a 3,4-linked mannose residue as a branch point.

Partial hydrolysis. The products obtained from partial hydrolysis of B62 EPS were separated on a Biogel P2 column. A fraction containing a pure oligosaccharide was isolated and investigated by the use of one-dimensional and two-dimensional NMR spectroscopies. The chemical shifts (Table 2) were compatible with the sequence 4-*O*-L-Lac-β-D-Glc*p*A-(1→4)-L-Fuc, where Lac is lactate and Glc*p*A is glucuronic acid.

Oligosaccharide CL1. The degradation of the deacetylated B62 EPS with the cellulase from *P. funiculosum*, followed by gel-permeation chromatography, yielded only oligosaccharide CL1, while no residual EPS was recovered, indicating that the reaction went to completion. The ion-spray MS spectrum of the oligosaccharide (Fig. 1), recorded in the negative ion mode, showed two $[M-1]^-$ ions, one at 735.3 Da, and the other at 757.4 Da. They were assigned to a tetrasaccharide composed of one carboxyethylhexuronic acid residue, one deoxyhexose residue and two hexose residues, in the acid form (*m/z* 735.3) and with one Na^+ as counter ion (*m/z* 757.4), respectively.

The CL1 oligosaccharide was reduced with NaB^2H_4, then methylated, hydrolysed and the products were derivatised to alditol acetates. GLC/MS analysis showed the presence of 4-linked glucitol (deuterated in C1), 4-linked fucopyranosyl, 3-linked mannopyranosyl, and 3-linked glucopyranosyl residues (Table 1). A sample of methylated CL1 was treated with $LiAl^2H_4$ to reduce the methyl ester groups. GLC/MS analysis of the derived alditol acetates revealed the presence of a new component corresponding to 4-*O*-lactyl-glucopyranosiduronic acid (Table 1). The results showed that the cellulase cleaved the β(1→4) linkage between the glucosyl residue and the branched residue, thus indicating a non-discriminating action of the enzyme towards the presence of 3,4-linked glucose or 3,4-linked

Table 2. ^1H and ^{13}C chemical shifts of 4-*O*-L-Lac-β-D-Glc*p*A-(1→4)-L-Fuc. The chemical shifts are given relative to internal acetone set to 2.225 ppm and 31.07 ppm for ^1H and ^{13}C, respectively.

Residue	Nucleus	Proton or carbon					
		1	2	3	4	5	6
		ppm					
-4)GlcA(β1-	H	4.49	3.48	3.65	3.37	3.77	
	C	103.5	74.3	75.1	81.9	76.7	175.9
-4)Fuc(α1-	H	5.23	3.78	3.78	3.99	4.28	1.29
	C	93.1	69.4	69.5	81.9	67.3	16.2
-4)Fuc(β1-	H	4.59	3.45	3.60	3.93	3.87	1.33
	C	97.0	73.0	73.0	81.0	71.6	16.2
Lac	H	1.33	3.94				
	C	19.7	79.1	182.7			

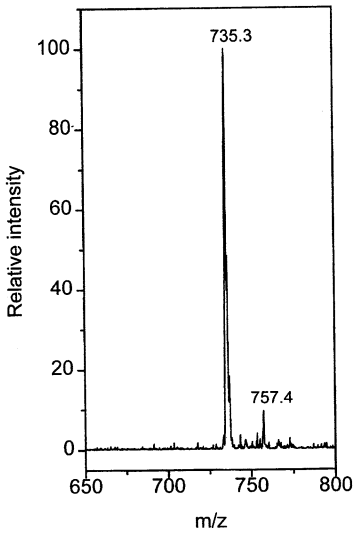


Fig. 1. Negative-mode ion-spray mass spectrum of CL1.

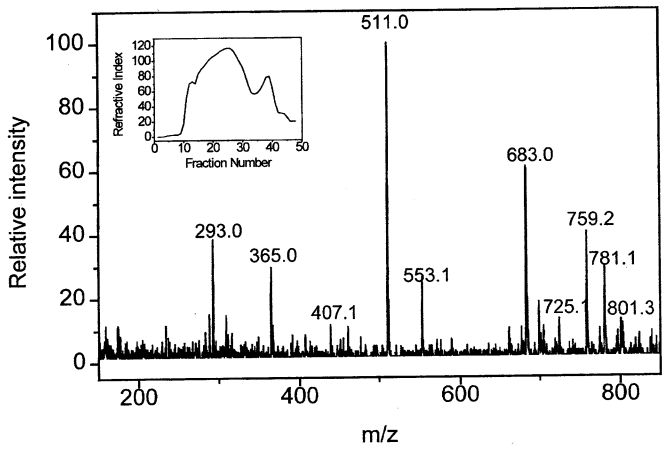


Fig. 2. Positive-mode collision-induced dissociation (orifice voltage 150 V, ion-spray voltage 5000 V) of the mixture of CL1 and CL1-Ac. In the inset the Biogel P2 elution profile of the products obtained upon treatment of B62 EPS with cellulase is shown.

mannose. Moreover, the data reported indicated that CL1 is a mixture of two tetrasaccharides, differing from each other by one sugar residue.

The absolute configuration of the sugar residues was determined on a sample of the CL1 oligosaccharide, which was subjected to methanolysis, treatment with boron tribromide to remove the lactyl substituent, and butanolysis. The configuration was shown to be L for the fucose residues and D for all the other sugars.

Location of the *O*-acetyl substituents. A sample of B62 EPS was treated with cellulase from *P. funiculosum* and purified as described above. The elution profile obtained upon separation of the products on a Biogel P2 column was very different from the one obtained when the deacetylated B62 EPS was used. It showed two main peaks, one at the exclusion volume and one with a retention time corresponding to the tetrasaccharide CL1, both not separated from a very broad band that eluted throughout the range of the inclusion volume of the column (Fig. 2). Positive-ion-mode ion-spray MS of single fractions located the tetrasaccharides corresponding to non acetylated CL1 and acetylated CL1 (CL1-Ac). The fraction containing a high amount of CL1-Ac (fraction 34) was subjected to ion-spray MS analysis in the positive-ion mode, and the tetrasaccharide was fragmented by increasing the orifice voltage to 150 V (Fig. 2). The fragmentation pattern established the sequence Lac-HexA-dHex-Hex-Hex, where Hex is hexose, dHex is deoxyhexose and HexA is hexuronic acid, which is in agreement with the results from methylation analysis, enzymatic degradation and partial acid hydrolysis, and indicated that the *O*-acetyl substituents are located on the hexopyranosyl residues of the backbone (Table 3). Fractions 16–40 were pooled, freeze/dried and methylated under neutral conditions (Prehm, 1980). The results of the GLC/MS analysis of the derived alditol acetates indicated that *O*-acetyl substituents are present on 75% of the repeating units, and that they are linked to C6 of all the mannopyranosyl residues and to part of C6 of the glucopyranosyl residues. These results were corroborated by inspection of the ^{13}C-NMR spectrum of B62 EPS (data not shown).

NMR studies of the oligosaccharide CL1. The ^1H-NMR spectrum of CL1 (Fig. 3) showed resonances attributable to three α (5.31, 5.22 and 5.09 ppm) and four β (4.75, 4.66, 4.52 and 4.49 ppm) anomeric protons, which were named a–g in order of decreasing chemical shift. Integration data indicated that CL1 is a tetrasaccharide, although the area for each of the anomeric signals was less than one, except for resonance g, which resulted after deconvolution of the overlapping signals f and g (Pons et al., 1996). The signals at 5.22 ppm (b) and 4.66 ppm (e) were

Table 3. Assignment of the ions obtained upon collision-induced dissociation of the mixture containing CL1 and CL1-Ac (fraction 34). Assignments of ions in Fig. 2 not reported in the Table were not determined. (Na), Na^+ was present as counterion.

m/z	Proposed composition
801.3	[Lac-GlcA-Fuc-Hex-Hex-OAc + Na]$^+$
781.1	[Lac-GlcA(Na)-Fuc-Hex-Hex + Na]$^+$
759.2	[Lac-GlcA-Fuc-Hex-Hex + Na]$^+$
553.1	[Fuc-Hex-Hex-OAc + Na]$^+$
551.0	[Fuc-Hex-Hex + Na]$^+$
407.1	[Hex-Hex-OAc + Na]$^+$
365.0	[Hex-Hex + Na]$^+$

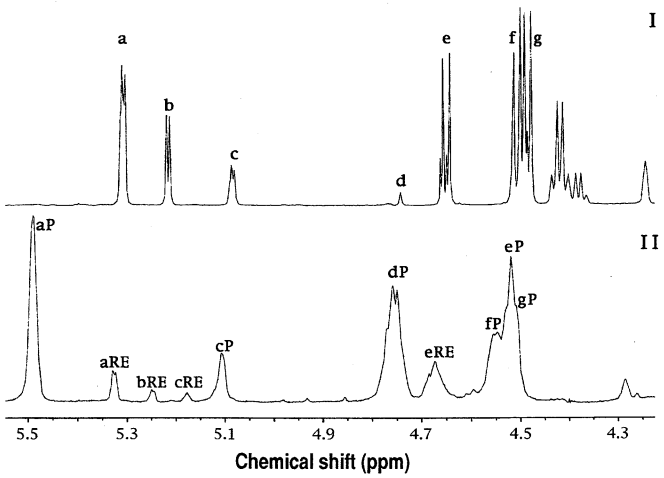


Fig. 3. Anomeric region of the 600-MHz ^{1}H-NMR spectra. (I), oligosaccharide CL1 obtained by cellulase digestion of the *P. flavescens* deacetylated B62 EPS; (II), deacetylated B62 EPS. Anomeric resonances were named a−g in order of decreasing chemical shift in (I). The same nomenclature was mantained in (II), with the addition of the suffixes P or RE for the residues of repeating units internal or at the reducing end, respectively.

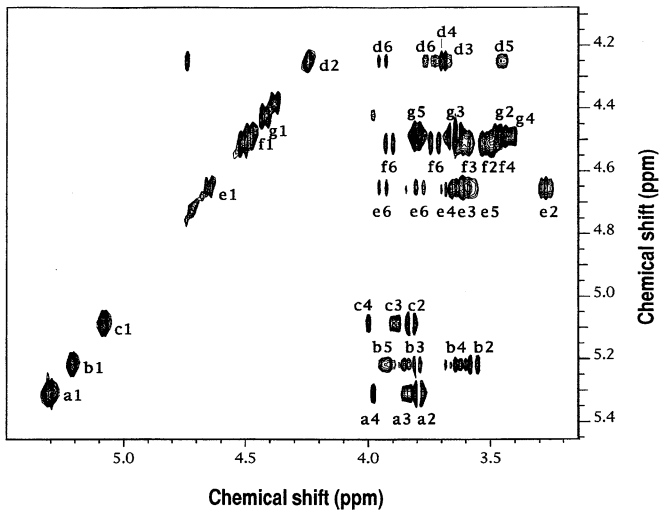


Fig. 4. Expansion of the 400-MHz TOCSY contour plot of the oligosaccharide CL1 obtained from a 512×512 data matrix, using a mixing time (τ_m) of 120 ms. The proton labelling at the cross-peaks refers to direct or remote correlation of the protons in each spin system with the anomeric protons.

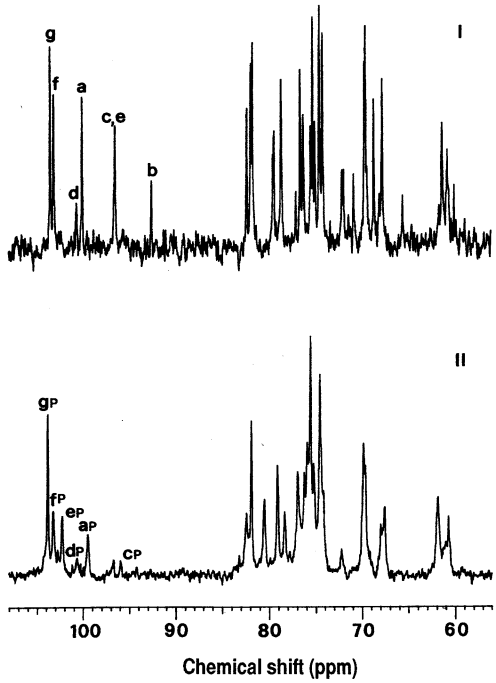


Fig. 5. Expansion of the 50-MHz ^{13}C NMR spectra of (I) oligosaccharide CL1 and (II) deacetylated B62 EPS.

assigned to the α- and β-glucopyranosyl reducing ends, respectively, and their assignments were confirmed by inspection of the HSQC contour plot. The resonances at 5.31 ppm and 5.09 ppm, which gave a peak area ratio of 3:1, were attributed to H1 of α-fucopyranosyl units. This finding is in agreement with the methylation analysis results that indicated the existence of two different residues as branch points, glucose and mannose. The integral of the H1 signal for residue d, partially suppressed with the residual water, was estimated in a separate experiment performed at 60 °C (spectrum not shown) and was 0.25, equal to the peak area of both H2 mannose at 4.26 pm (Fig. 3) and H1 fucose at 5.09 ppm. The methyl doublets at 1.28 ppm and 1.26 ppm confirmed the presence of the fucose residues, while that one at 1.34 ppm was assigned to the carboxyethyl substituent.

TOCSY experiments of CL1 afforded the assignment of most of the proton resonances (Fig. 4). Although H1 (4.75 ppm) of the d residue was suppressed, the complete unravelling of its spin system was achieved starting from H2.

The ^{13}C-NMR spectrum of CL1 (Fig. 5) showed six C1 peaks in the region 104−91 ppm and their assignment followed from inspection of the HSQC diagram, which revealed two overlapping signals at 96.5 ppm belonging to the β-anomeric carbon of the glucopyranosyl unit reducing end and to C1 of a fucose residue (c). The signal at 92.6 ppm was assigned to the α anomeric carbon of the glucopyranosyl unit reducing end. The signal at 100.6 ppm was assigned to C1 of the mannose residue (d) and its $J_{C1,\,H1}$ value (158.5 Hz) was indicative of a β-linkage (Bock et al., 1973). The carbon resonances at 19.6 ppm and 181.9 ppm (signals not shown) assigned to methyl and carboxyl groups, respectively, confirmed the presence of the lactyl substituent (Parolis et al., 1988).

The HSQC plot of CL1 (Fig. 6) completed the assignment of the remaining signals and confirmed the position of the linkages for each residue. The ^{1}H and ^{13}C chemical shifts for the oligosaccharide CL1 are reported in Table 4.

The HMQC experiment of CL1 (Fig. 7), optimised for long-range couplings (Hurd and John, 1991), confirmed some of the

Table 4. ^{1}H and ^{13}C chemical shifts of the tetrasaccharide CL1. The chemical shifts are given relative to internal acetone set to 2.225 ppm and 31.07 ppm for ^{1}H and ^{13}C, respectively. The residues were named a−g in order of decreasing chemical shift.

Residue		Nucleus	Proton or carbon					
			1	2	3	4	5	6
			ppm					
-4)Fuc(α1-	(a)	H	5.31	3.83	3.89	4.00	4.43	1.26
		C	100.0	69.6	69.7	81.9	67.8	15.8
-4)Glcα	(b)	H	5.22	3.57	3.83	3.63	3.92	3.80−3.94
		C	92.5	71.9	72.1	79.6	70.8	60.8
-4)Fuc(α1-	(c)	H	5.09	3.84	3.90	4.00	4.43	1.28
		C	96.5	69.6	69.7	81.9	67.8	15.8
-3)Man(β1-	(d)	H	4.75	4.25	3.70	3.69	3.46	3.77−3.95
		C	100.6	68.0	78.7	65.6	77.1	61.7
-4(Glcβ	(e)	H	4.66	3.28	3.63	3.67	3.60	3.80−3.95
		C	96.5	74.6	75.1	79.4	75.5	60.9
-3)Glc(β1-	(f)	H	4.52	3.50	3.62	3.52	3.52	3.75−3.94
		C	103.1	74.6	82.3	68.7	76.3	61.3
-4GlcA(β1-	(g)	H	4.49	3.46	3.67	3.42	3.82	
		C	103.5	74.2	75.3	81.7	76.6	175.4
Lac		H	1.34	4.02				
		C	19.5	78.9	181.5			

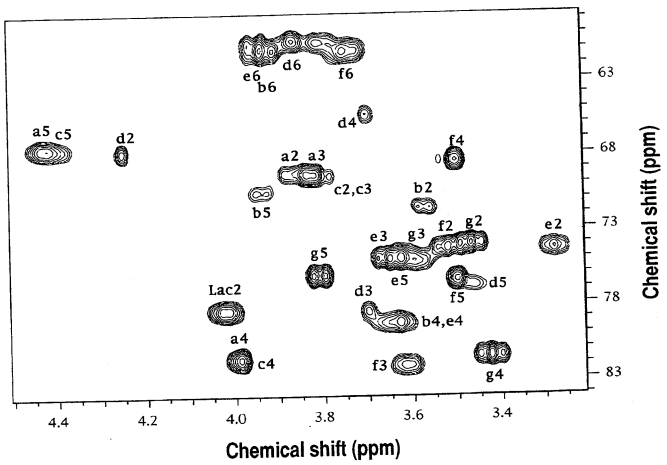


Fig. 6. Part of the HSQC contour plot of the oligosaccharide CL1. ^{1}H-^{13}C chemical shifts correlation peaks are labelled with their assignments (Table 4).

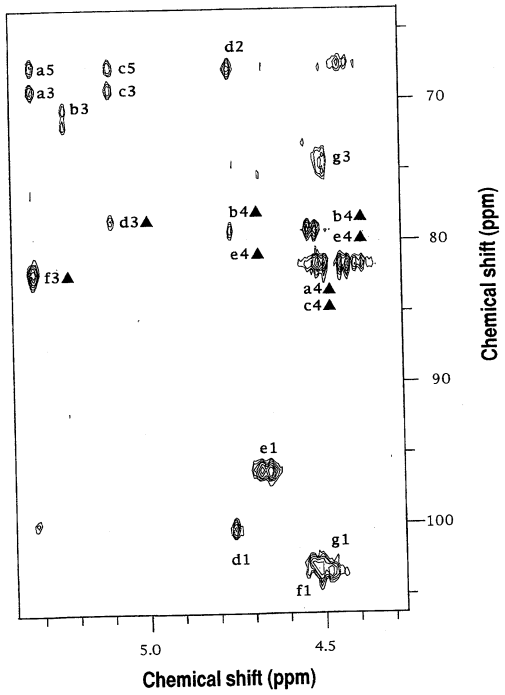


Fig. 7. Part of the gradient-enhanced HMQC contour plot of the oligosaccharide CL1. The HMQC experiment was optimised for the determination of long-range ^{1}H-^{13}C connectivities and the delay for the evolution of $^{3}J_{C,H}$ couplings was calculated from a value of 7 Hz. ▲ interresidue connectivities indicating aglycone carbons.

assignments and established the following inter-residue linkages: a(1→3)f; c(1→3)d; d(1→4)b; d(1→4)e; f(1→4)b; f(1→4)e; g(1→4)a; and g(1→4)c.

The results indicate that CL1 is a mixture of two tetrasaccharides with the common following structure:

4-*O*-L-Lac-β-D-Glc*p*A-(1→4)-α-L-Fuc*p*-(1→3)-β-D-X*p*-(1→4)-D-Glc

where X is either glucose or mannose in a ratio 3:1.

NMR studies of the deacetylated B62 EPS. The ^{1}H-NMR spectrum of the deacetylated B62 EPS (Fig. 3) showed five resonances in the region 5.6−5.0 ppm typical of α-anomeric protons. From the NMR studies of oligosaccharide CL1 it is evident that fucose is the only residue with an α configuration. The resonances at 5.49 ppm and 5.11 ppm were assigned to H1 of fucose linked to glucose (aP) and mannose (cP), respectively, whereas the resonances at 5.33 ppm and 5.18 ppm were attributed to H1 of fucose linked to the two branch-point residues adjacent to the reducing end (aRE and cRE). This latter assignment is confirmed by the signal at 5.25 ppm due to H1 of the α glucopyranosyl reducing end. Integration data showed that the ratios between the peak areas of aP and cP, and aRE and cRE

Table 5. ^{1}H and ^{13}C chemical shifts of the deacetylated B62 EPS. The chemical shifts related to the reducing and non-reducing ends are not reported. The residues were named aP to gP to distinguish them from the residues in the tetrasaccharide CL1. The chemical shifts are given relative to internal acetone set equal to 2.225 ppm and 31.07 ppm for ^{1}H and ^{13}C, respectively.

Residue		Nucleus	Proton or carbon					
			1	2	3	4	5	6
			ppm					
-4)Fuc(α1-	(aP)	H	5.48	3.86	3.96	3.99	4.76	1.30
		C	99.4	69.8	69.7	82.4	67.6	16.1
-4)Fuc(α1-	(cP)	H	5.09	3.86	3.96	3.99	4.67	1.30
		C	95.9	69.8	69.7	82.4	67.6	16.1
-3,4)Man(β1-	(dP)	H	4.76	4.29	3.84	3.86	3.60	3.71−4.03
		C	100.6	68.1	78.3	74.1		61.9
-4)Glc(β1-	(eP)	H	4.51	3.29	3.67	3.51	3.53	3.86−4.03
		C	102.2	74.5	75.2	80.5	75.9	60.7
-3,4)Glc(β1-	(fP)	H	4.54	3.56	3.66	3.53	3.53	3.86−3.97
		C	103.2	74.5				60.9
-4)GlcA(β1-	(gP)	H	4.50	3.51	3.67	3.43	3.78	
		C	103.8	74.5	75.5	81.8	76.9	175.4
Lac		H	1.37	4.06				
		C	19.8	79.1	182.1			

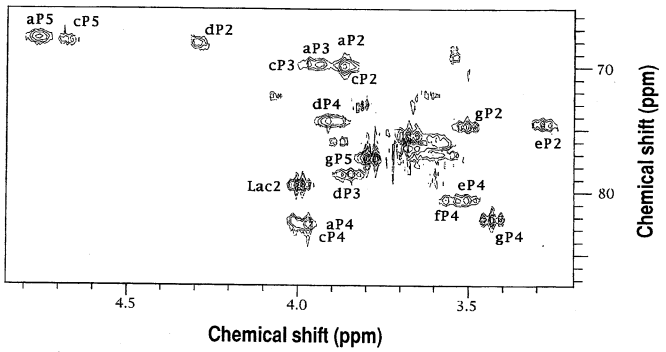


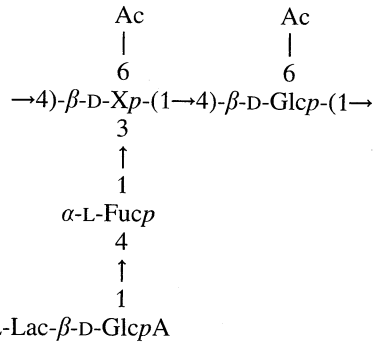
Fig. 8. Part of the HSQC contour plot of the deacetylated B62 polysaccharide. ^{1}H-^{13}C chemical shift correlation peaks are labelled with their assignments (Table 5).

are equal to 3:1, which is the same value found for the fucose residues in the CL1 oligosaccharide. Moreover, the comparison of the peak areas of aP and aRE gave a ratio of 8:1, suggesting an average degree of polymerisation of nine repeating units. These results are in agreement with the low molecular mass (9000 Da) exhibited by B62 EPS. The strong signal overlap in the region 4.8−4.4 ppm prevented the assignment of the β-anomeric protons, which on the contrary were clearly identified in the HSQC experiment. The assignment of most of the ^{1}H resonances derived from TOCSY experiments.

The ^{13}C-NMR spectrum of the deacetylated B62 EPS (Fig. 5) showed four main resonances in the anomeric region and some low-intensity signals, which were assigned after inspection of the HSQC plot. Comparison of the HSQC plot of CL1 with that of deacetylated B62 EPS (Fig. 8) identified the cross-peaks of the repeating units in the polymer, and those at the non-reducing and reducing ends. The HMBC experiment of deacetylated B62 EPS (plot not shown) confirmed some of the assignments and independently established the inter-residue linkages. The complexity of the deacetylated B62 EPS spectra, due to its low molecular mass and to the presence of two repeating units differing for the sugar at the branch point, prevented the complete assignment of the chemical shifts. The ^{1}H and ^{13}C chemical shifts for the internal repeating units of deacetylated B62 EPS are reported in Table 5.

DISCUSSION

P. flavescens strain B62 was shown to produce a mixture of two polysaccharides, differing in composition and M_w. The production of two EPS by the same bacterium was reported previously for the genus *Pseudomonas* and also for other genera, such as *Agrobacterium*, *Clavibacter* and *Erwinia* (Fett, 1993). Our attention was focussed on the most abundant polysaccharide (B62 EPS), which has the following structure, on the basis of the collected data:



where X is glucose (75%) or mannose (25%). 75% of the repeating units are acetylated on the C6 of the hexose residues in non-stoichiometric amounts.

The primary structure of this polymer is rather unusual in three ways; the presence of a lactyl substituent; the partial but constant replacement of the branched glucose with a branched mannose residue; and its low molecular mass. The lactyl group has been found in bacterial polysaccharides, mostly attached to a neutral sugar (Kochetkov et al., 1979; Jansson et al., 1984; Osman and Fett, 1993; Osman et al., 1994; Garozzo et al.,

1995), rather than to a glucuronic acid residue (Parolis et al., 1988; Lindberg et al., 1976), and it is not as common as pyruvyl or acetyl groups. The replacement of branched glucose by branched mannose residues in the repeating units is unusual. The distribution of these two repeating segments in the chain was not established, partly due to the lack of discrimination by the fungal cellulase. In principle, several possibilities exist. In the simplest case a distribution, according to unknown statistics, of the branched mannose and glucose residues within the same chain may occur (e.g. resembling the complex distribution pattern of guluronic acid and mannuronic acid residues in algal alginate). In contrast, a distribution of the two sugars in two sets of chains could be envisaged; however, intuitive biosynthetic considerations render such a hypothesis quite remote. The presence of two types of repeating units differing from each other for one sugar residue has been described, namely in the gellan-gum family of polysaccharides, where an α-L-mannopyranosyl residue partially replaces the α-L-rhamnopyranosyl residue either in the main chain [S-88 (Jansson et al., 1986) and S-198 (Chowdhury et al., 1987)] or as a single-unit side chain [S-130 (Jansson et al., 1985)]. The low molecular mass of the present EPS is a rather uncommon feature, since most known bacterial polysaccharides exhibit molecular masses of about 1 MDa.

A key step in the structural determination of this EPS was the use of a specific enzyme to obtain oligosaccharides corresponding to the repeating unit. The presence of the 4-*O*-L-Lac-β-D-Glc*p*A non-reducing terminus in the side chain suggested the use of bacteriophage φ22 endoglycanase (Parolis et al., 1988), since it was very effective in cleaving the *Klebsiella pneumoniae* K22 polysaccharide, which also possesses a tetrasaccharidic repeating unit with the same non-reducing terminus in the side chain. However, bacteriophage φ22 endoglycanase was totally ineffective on B62 EPS. Although the carboxylate residue is very important for the substrate specificity of this type of endoglycanases, it is probably not a sufficient structural condition, the other strict requirements being the position of the hydroxyl groups and the sterical arrangement (Geyer et al., 1983). On the contrary, the cellulase from *P. funiculosum* cleaved very efficiently the β(1−4) glycosidic linkages adjacent to the side chain. Moreover, the presence of mannose did not prevent the action of this enzyme. Another important feature of the cellulase is that the depolymerisation to the repeating units was complete only when B62 EPS had been de-*O*-acetylated previously. Treatment of the native polymer with cellulase led to the production of oligosaccharides consisting of multiples of the repeating unit, with and without *O*-acetyl groups. A hypothetical role for this substituent could be the protection of the EPS from the action of microbial endoglucanases. It has been reported that *Xanthomonas campestris* pv *campestris* produces endoglucanase (Gough et al., 1988), and this could be true also for *Xanthomonas campestris* pv *juglandis* (causal agent of walnut blight canker), although no such data are available. Several plant pathogenic bacteria produce endoglucanases, which are probably involved in the degradation of plant cell-wall glucans, thus facilitating the bacterial penetration into the plant tissues and /or help in the release of plant cell components that could be used as nutrients (Gough et al., 1988; Roberts et al., 1988; Boccara et al., 1994).

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REFERENCES

Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography, *Carbohydr. Res. 5*, 340−345.

Åman, P., Franzèn, L.-E., Darwill, J. E., McNeil, M., Darvill, A. G. & Albersheim, P. (1982) The structure of the acidic polysaccharide secreted by *Rhizobium phaseoli* strain 127 K38, *Carbohydr. Res. 103*, 77−100.

Amemura, A., Harada, T., Abe, M. & Higashi, S. (1983) Structural studies on the extracellular acidic polysaccharide from *Rhizobium trifolii* 4S, *Carbohydr. Res. 115*, 165−174.

Bax, A. & Summers, M. F. (1986) ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR, *J. Am. Chem. Soc. 108*, 2093−2094.

Boccara, M., Aymeric, J. & Camus, C. (1994) Role of endoglucanases in *Erwinia chrysanthemi* 3937 virulence on *Saintpaulia ionantha*, *J. Bacteriol. 176*, 1524−1526.

Bock, K., Lundt, I. & Pedersen, C. (1973) Assignment of anomeric structure to carbohydrates through geminal ^{13}C-H coupling constants, *Tetrahedron Lett. 13*, 1037−1040.

Bodenhausen, G. & Ruben, D. J. (1980) Natural abudance nitrogen-15 NMR by enhanced heteronuclear spectroscopy, *Chem. Phys. Lett. 69*, 185−189.

Chowdhury, T. A., Lindberg, B., Lindquist, U. & Baird, J. (1987) Structural studies of an extracellular polysaccharide (S-198) elaborated by *Alcaligenes* ATCC 31853, *Carbohydr. Res. 161*, 127−132.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. (1995) Microbial biofilms, *Annu. Rev. Microbiol. 49*, 711−745.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) Colorimetric method for determination of sugars and related substances, *Anal. Chem. 28*, 350−356.

Fett, W. F. (1993) Bacterial exopolysaccharides: their nature, regulation and role in host-pathogen interactions, *Curr. Top. Bot. Res. 1*, 367−390.

Fett, W. F., Osman, S. F., Wijey, C. & Singh, S. (1993) Exopolysaccharides of rRNA group I pseudomonads, in *Carbohydrates and carbohydrate polymers* (Yalpani, M., ed.) pp. 52−61, ATL Press, Inc., M Prospect IL.

Fett, W. F., Cescutti, P. & Wijey, C. (1996) Exopolysaccharides of th plant pathogens *Pseudomonas corrugata* and *Ps. flavescens* and th saprophyte *Ps. chlororaphis*, *J. Appl. Bacteriol. 81*, 181−187.

Gacesa, P. & Russell, N. J. (1990) Pseudomonas *infection and alginates Biochemistry, genetics and pathology*, Chapman and Hall, London.

Garozzo, D., Impallomeni, G., Spina, E., Sturiale, L., Cesàro, A. & Cescutti, P. (1995) Identification of *N*-acetylglucosamine and 4-*O* [1-carboxyethyl]mannose in the exopolysaccharide from *Cyanospir capsulata*, *Carbohydr. Res. 270*, 97−106.

Geyer, H., Himmelspach, K., Kwiatkowski, B., Schlecht, S. & Stirn S. (1983) Degradation of bacterial surface carbohydrates by viru associated enzymes, *Pure Appl. Chem. 55*, 637−653.

Gerwig, G. J., Kamerling, J. P. & Vliegenthart, J. F. G. (1978) Determination of the D and L configuration of neutral monosaccharides b high-resolution capillary G.L.C., *Carbohydr. Res. 62*, 349−357.

Gerwig, G. J., Kamerling, J. P. & Vliegenthart, J. F. G. (1979) Determination of the absolute configuration of monosaccharides in compl carbohydrates by capillary G.L.C., *Carbohydr. Res. 77*, 1−7.

Gough, C. L., Dow, J. M., Barber, C. E. & Daniels, M. J. (1988) Cloni of two endoglucanase genes of *Xanthomonas campestris* p *campestris*: analysis of the role of the major endoglucanase in path genesis, *Mol. Plant-Microbe Interact. 1*, 275−281.

Hakomori, S. (1964) A rapid permethylation of glycolipid, and pol saccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfo ide, *J. Biochem. (Tokyo) 55*, 205−208.

Hildebrand, D. C., Palleroni, N. J., Hendson, M., Toth, J. & Johnson, J. L. (1994) *Pseudomonas flavescens* sp. nov., isolated from walnut blight cankers, *Int. J. System. Microbiol. 44*, 410–415.

Hough, L. & Theobald, R. S. (1963) De-*O*-methylation with hydrobromic acid and boron tribromide, *Methods Carbohydr. Chem. 2*, 203–206.

Hurd, R. E. & John, B. K. (1991) Gradient-enhanced proton-detected heteronuclear multiple-quantum coherence spectroscopy, *J. Magn. Reson. 91*, 648–653.

Jansson, P.-E., Lindberg, B., Lönngren, J., Ortega, C. & Nimmich, W. (1984) Structural studies of the capsular polysaccharide of *Klebsiella* type 66, *Carbohydr. Res. 132*, 297–305.

Jansson, P.-E., Lindberg, B., Widmalm, G. & Sandford, P. A. (1985) Structural studies of an extracellular polysaccharide (S-130) elaborated by *Alcaligenes* ATCC 31555, *Carbohydr. Res. 139*, 217–223.

Jansson, P.-E., Kumar, N. S. & Lindberg, B. (1986) Structural studies of a polysaccharide (S-88) elaborated by *Pseudomonas* ATCC 31554, *Carbohydr. Res. 156*, 165–172.

Kenne, L. & Lindberg, B. (1983) Bacterial polysaccharides, in *The polysaccharides* (Aspinall, G. O., ed.) vol. 2, pp. 287–363, Academic Press, New York.

Kochetkov, N. K., Sviridov, A. F., Arifkhodzhaev, K. A., Chizhov, O. S. & Shashkov, A. S. (1979) The structure of the extracellular polysaccharide from *Mycobacterium lacticolum* strain 121, *Carbohydr. Res. 71*, 193–203.

Leigh, J. A. & Coplin, D. L. (1992) Exopolysaccharides in plant-bacterial interactions, *Annu. Rev. Microbiol. 46*, 307–346.

Lindberg, B., Lindqvist, B. & Lönngren, J. (1976) 4-*O*-[(*S*)-1-Carboxyethyl]-D-glucuronic acid: a component of the *Klebsiella* type 37 capsular polysaccharide, *Carbohydr. Res. 49*, 411–417.

Osman, S. F. & Fett, W. F. (1993) Structure of the acidic exopolysaccharide of *Pseudomonas marginalis* strain ATCC 10844, *Carbohydr. Res. 242*, 271–275.

Osman, S. F., Fett, W. F. & Dudley, R. L. (1994) Structure of the exopolysaccharide of *Pseudomonas stutzeri* strain ATCC 17588, *Carbohydr. Res. 265*, 319–322.

Palleroni, N. J. (1993) *Pseudomonas* classification: a new case history in the taxonomy of Gram-negative bacteria, *Antonie Leeuwenhoek 64*, 231–251.

Parolis, L. A., Parolis, H., Niemann, H. & Stirm, S. (1988) Primary structure of *Klebsiella* serotype K22 capsular polysaccharide: another glycan containing 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid, *Carbohydr. Res. 179*, 301–314.

Phillips, L. R. & Fraser, B. A. (1981) Methylation of carbohydrates with dimsyl potassium in dimethyl sulfoxide, *Carbohydr. Res. 90*, 149–152.

Pons, J. L., Malliavin, T. E. & Delsuc, M. A. (1996) Gifa V4: a complete package for the NMR data-set processing, *J. Biomol. NMR 8*, 445–452.

Prehm, P. (1980) Methylation of carbohydrates by methyl trifluoromethanesulfonate in trimethyl phosphate, *Carbohydr. Res. 78*, 372–374.

Roberts, D. P., Denny, T. P. & Schell, M. A. (1988) Cloning of the *egl* gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity, *J. Bacteriol. 170*, 1445–1451.

Sanford, P. A. & Baird, J. (1983) Industrial utilization of polysaccharides, in *The polysaccharides* (Aspinall, G. O., ed.) vol. 2, pp. 411–485, Academic Press, New York.

Sweet, D. P., Shapiro, R. H. & Albersheim, P. (1975) Quantitative analysis by various g.l.c. response-factor theories for partially methylated and partially ethylated alditol acetates, *Carbohydr. Res. 40*, 217–225.

Taylor, R. L. & Conrad, H. E. (1972) Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups, *Biochemistry 11*, 1383–1388.

Table 3. Assignment of the ions obtained upon collision-induced dissociation of the mixture containing CL1 and CL1-Ac (fraction 34). Assignments of ions in Fig. 2 not reported in the Table were not determined. (Na), Na^+ was present as counterion.

m/z	Proposed composition
801.3	[Lac-GlcA-Fuc-Hex-Hex-OAc + Na]$^+$
781.1	[Lac-GlcA(Na)-Fuc-Hex-Hex + Na]$^+$
759.2	[Lac-GlcA-Fuc-Hex-Hex + Na]$^+$
553.1	[Fuc-Hex-Hex-OAc + Na]$^+$
551.0	[Fuc-Hex-Hex + Na]$^+$
407.1	[Hex-Hex-OAc + Na]$^+$
365.0	[Hex-Hex + Na]$^+$

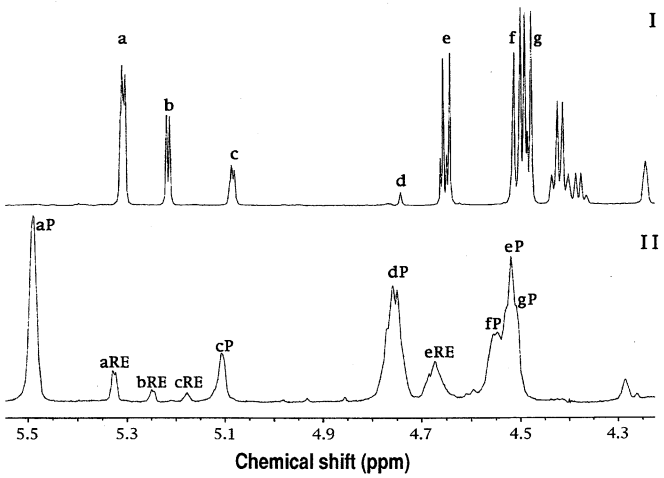


Fig. 3. Anomeric region of the 600-MHz ^{1}H-NMR spectra. (I), oligosaccharide CL1 obtained by cellulase digestion of the *P. flavescens* deacetylated B62 EPS; (II), deacetylated B62 EPS. Anomeric resonances were named a−g in order of decreasing chemical shift in (I). The same nomenclature was mantained in (II), with the addition of the suffixes P or RE for the residues of repeating units internal or at the reducing end, respectively.

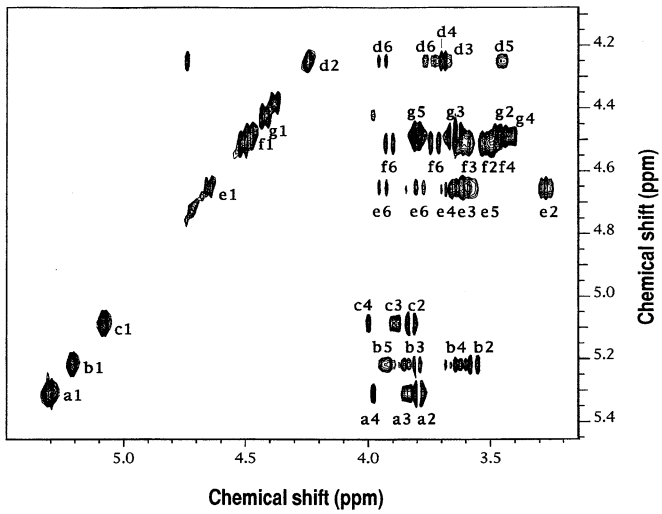


Fig. 4. Expansion of the 400-MHz TOCSY contour plot of the oligosaccharide CL1 obtained from a 512×512 data matrix, using a mixing time (τ_m) of 120 ms. The proton labelling at the cross-peaks refers to direct or remote correlation of the protons in each spin system with the anomeric protons.

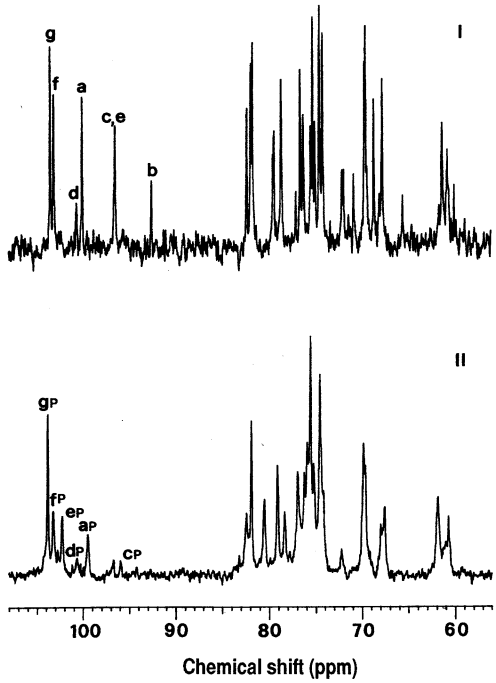


Fig. 5. Expansion of the 50-MHz ^{13}C NMR spectra of (I) oligosaccharide CL1 and (II) deacetylated B62 EPS.

assigned to the α- and β-glucopyranosyl reducing ends, respectively, and their assignments were confirmed by inspection of the HSQC contour plot. The resonances at 5.31 ppm and 5.09 ppm, which gave a peak area ratio of 3:1, were attributed to H1 of α-fucopyranosyl units. This finding is in agreement with the methylation analysis results that indicated the existence of two different residues as branch points, glucose and mannose. The integral of the H1 signal for residue d, partially suppressed with the residual water, was estimated in a separate experiment performed at 60 °C (spectrum not shown) and was 0.25, equal to the peak area of both H2 mannose at 4.26 pm (Fig. 3) and H1 fucose at 5.09 ppm. The methyl doublets at 1.28 ppm and 1.26 ppm confirmed the presence of the fucose residues, while that one at 1.34 ppm was assigned to the carboxyethyl substituent.

TOCSY experiments of CL1 afforded the assignment of most of the proton resonances (Fig. 4). Although H1 (4.75 ppm) of the d residue was suppressed, the complete unravelling of its spin system was achieved starting from H2.

The ^{13}C-NMR spectrum of CL1 (Fig. 5) showed six C1 peaks in the region 104−91 ppm and their assignment followed from inspection of the HSQC diagram, which revealed two overlapping signals at 96.5 ppm belonging to the β-anomeric carbon of the glucopyranosyl unit reducing end and to C1 of a fucose residue (c). The signal at 92.6 ppm was assigned to the α anomeric carbon of the glucopyranosyl unit reducing end. The signal at 100.6 ppm was assigned to C1 of the mannose residue (d) and its $J_{C1,\,H1}$ value (158.5 Hz) was indicative of a β-linkage (Bock et al., 1973). The carbon resonances at 19.6 ppm and 181.9 ppm (signals not shown) assigned to methyl and carboxyl groups, respectively, confirmed the presence of the lactyl substituent (Parolis et al., 1988).

The HSQC plot of CL1 (Fig. 6) completed the assignment of the remaining signals and confirmed the position of the linkages for each residue. The ^{1}H and ^{13}C chemical shifts for the oligosaccharide CL1 are reported in Table 4.

The HMQC experiment of CL1 (Fig. 7), optimised for long-range couplings (Hurd and John, 1991), confirmed some of the

Table 4. ^{1}H and ^{13}C chemical shifts of the tetrasaccharide CL1. The chemical shifts are given relative to internal acetone set to 2.225 ppm and 31.07 ppm for ^{1}H and ^{13}C, respectively. The residues were named a−g in order of decreasing chemical shift.

Residue		Nucleus	Proton or carbon					
			1	2	3	4	5	6
			ppm					
-4)Fuc(α1-	(a)	H	5.31	3.83	3.89	4.00	4.43	1.26
		C	100.0	69.6	69.7	81.9	67.8	15.8
-4)Glcα	(b)	H	5.22	3.57	3.83	3.63	3.92	3.80−3.94
		C	92.5	71.9	72.1	79.6	70.8	60.8
-4)Fuc(α1-	(c)	H	5.09	3.84	3.90	4.00	4.43	1.28
		C	96.5	69.6	69.7	81.9	67.8	15.8
-3)Man(β1-	(d)	H	4.75	4.25	3.70	3.69	3.46	3.77−3.95
		C	100.6	68.0	78.7	65.6	77.1	61.7
-4(Glcβ	(e)	H	4.66	3.28	3.63	3.67	3.60	3.80−3.95
		C	96.5	74.6	75.1	79.4	75.5	60.9
-3)Glc(β1-	(f)	H	4.52	3.50	3.62	3.52	3.52	3.75−3.94
		C	103.1	74.6	82.3	68.7	76.3	61.3
-4GlcA(β1-	(g)	H	4.49	3.46	3.67	3.42	3.82	
		C	103.5	74.2	75.3	81.7	76.6	175.4
Lac		H	1.34	4.02				
		C	19.5	78.9	181.5			

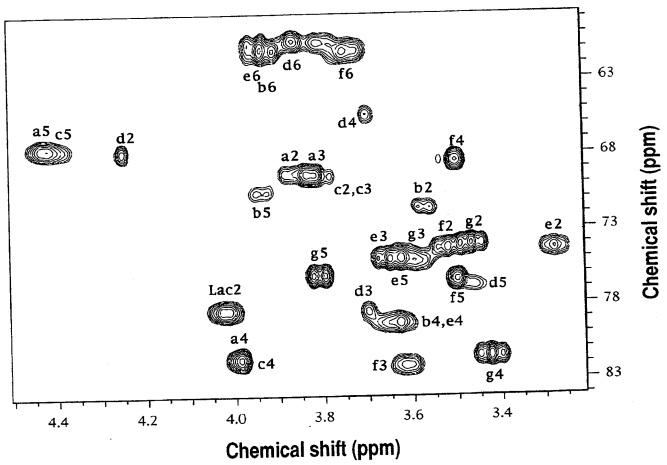


Fig. 6. Part of the HSQC contour plot of the oligosaccharide CL1. ^{1}H-^{13}C chemical shifts correlation peaks are labelled with their assignments (Table 4).

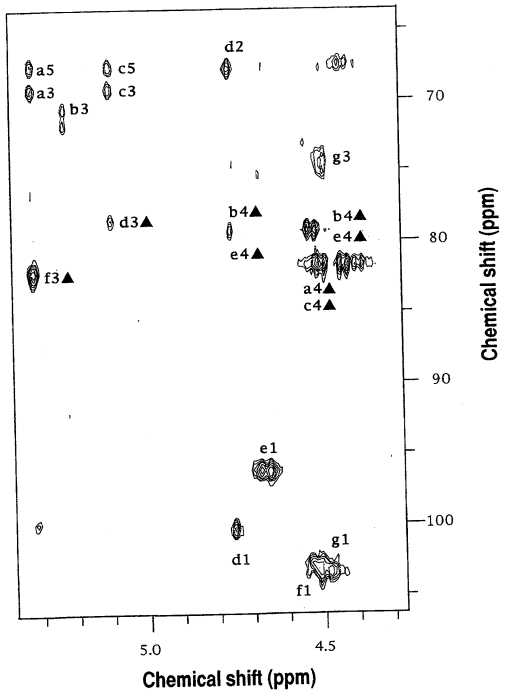


Fig. 7. Part of the gradient-enhanced HMQC contour plot of the oligosaccharide CL1. The HMQC experiment was optimised for the determination of long-range ^{1}H-^{13}C connectivities and the delay for the evolution of $^{3}J_{C,H}$ couplings was calculated from a value of 7 Hz. ▲ interresidue connectivities indicating aglycone carbons.

assignments and established the following inter-residue linkages: a(1→3)f; c(1→3)d; d(1→4)b; d(1→4)e; f(1→4)b; f(1→4)e; g(1→4)a; and g(1→4)c.

The results indicate that CL1 is a mixture of two tetrasaccharides with the common following structure:

4-*O*-L-Lac-β-D-Glc*p*A-(1→4)-α-L-Fuc*p*-(1→3)-β-D-X*p*-(1→4)-D-Glc

where X is either glucose or mannose in a ratio 3:1.

NMR studies of the deacetylated B62 EPS. The ^{1}H-NMR spectrum of the deacetylated B62 EPS (Fig. 3) showed five resonances in the region 5.6−5.0 ppm typical of α-anomeric protons. From the NMR studies of oligosaccharide CL1 it is evident that fucose is the only residue with an α configuration. The resonances at 5.49 ppm and 5.11 ppm were assigned to H1 of fucose linked to glucose (aP) and mannose (cP), respectively, whereas the resonances at 5.33 ppm and 5.18 ppm were attributed to H1 of fucose linked to the two branch-point residues adjacent to the reducing end (aRE and cRE). This latter assignment is confirmed by the signal at 5.25 ppm due to H1 of the α glucopyranosyl reducing end. Integration data showed that the ratios between the peak areas of aP and cP, and aRE and cRE

Table 5. ^{1}H and ^{13}C chemical shifts of the deacetylated B62 EPS. The chemical shifts related to the reducing and non-reducing ends are not reported. The residues were named aP to gP to distinguish them from the residues in the tetrasaccharide CL1. The chemical shifts are given relative to internal acetone set equal to 2.225 ppm and 31.07 ppm for ^{1}H and ^{13}C, respectively.

Residue		Nucleus	Proton or carbon					
			1	2	3	4	5	6
			ppm					
-4)Fuc(α1-	(aP)	H	5.48	3.86	3.96	3.99	4.76	1.30
		C	99.4	69.8	69.7	82.4	67.6	16.1
-4)Fuc(α1-	(cP)	H	5.09	3.86	3.96	3.99	4.67	1.30
		C	95.9	69.8	69.7	82.4	67.6	16.1
-3,4)Man(β1-	(dP)	H	4.76	4.29	3.84	3.86	3.60	3.71−4.03
		C	100.6	68.1	78.3	74.1		61.9
-4)Glc(β1-	(eP)	H	4.51	3.29	3.67	3.51	3.53	3.86−4.03
		C	102.2	74.5	75.2	80.5	75.9	60.7
-3,4)Glc(β1-	(fP)	H	4.54	3.56	3.66	3.53	3.53	3.86−3.97
		C	103.2	74.5				60.9
-4)GlcA(β1-	(gP)	H	4.50	3.51	3.67	3.43	3.78	
		C	103.8	74.5	75.5	81.8	76.9	175.4
Lac		H	1.37	4.06				
		C	19.8	79.1	182.1			

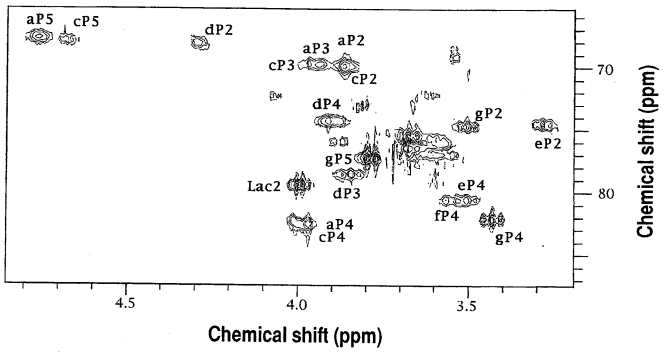


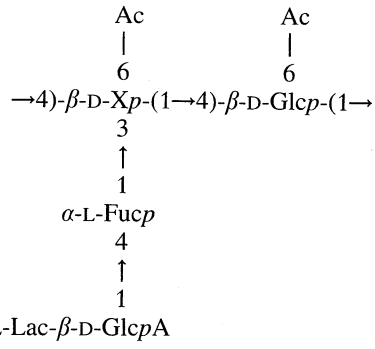
Fig. 8. Part of the HSQC contour plot of the deacetylated B62 polysaccharide. ^{1}H-^{13}C chemical shift correlation peaks are labelled with their assignments (Table 5).

are equal to 3:1, which is the same value found for the fucose residues in the CL1 oligosaccharide. Moreover, the comparison of the peak areas of aP and aRE gave a ratio of 8:1, suggesting an average degree of polymerisation of nine repeating units. These results are in agreement with the low molecular mass (9000 Da) exhibited by B62 EPS. The strong signal overlap in the region 4.8−4.4 ppm prevented the assignment of the β-anomeric protons, which on the contrary were clearly identified in the HSQC experiment. The assignment of most of the ^{1}H resonances derived from TOCSY experiments.

The ^{13}C-NMR spectrum of the deacetylated B62 EPS (Fig. 5) showed four main resonances in the anomeric region and some low-intensity signals, which were assigned after inspection of the HSQC plot. Comparison of the HSQC plot of CL1 with that of deacetylated B62 EPS (Fig. 8) identified the cross-peaks of the repeating units in the polymer, and those at the non-reducing and reducing ends. The HMBC experiment of deacetylated B62 EPS (plot not shown) confirmed some of the assignments and independently established the inter-residue linkages. The complexity of the deacetylated B62 EPS spectra, due to its low molecular mass and to the presence of two repeating units differing for the sugar at the branch point, prevented the complete assignment of the chemical shifts. The ^{1}H and ^{13}C chemical shifts for the internal repeating units of deacetylated B62 EPS are reported in Table 5.

DISCUSSION

P. flavescens strain B62 was shown to produce a mixture of two polysaccharides, differing in composition and M_w. The production of two EPS by the same bacterium was reported previously for the genus *Pseudomonas* and also for other genera, such as *Agrobacterium*, *Clavibacter* and *Erwinia* (Fett, 1993). Our attention was focussed on the most abundant polysaccharide (B62 EPS), which has the following structure, on the basis of the collected data:



where X is glucose (75%) or mannose (25%). 75% of the repeating units are acetylated on the C6 of the hexose residues in non-stoichiometric amounts.

The primary structure of this polymer is rather unusual in three ways; the presence of a lactyl substituent; the partial but constant replacement of the branched glucose with a branched mannose residue; and its low molecular mass. The lactyl group has been found in bacterial polysaccharides, mostly attached to a neutral sugar (Kochetkov et al., 1979; Jansson et al., 1984; Osman and Fett, 1993; Osman et al., 1994; Garozzo et al.,

1995), rather than to a glucuronic acid residue (Parolis et al., 1988; Lindberg et al., 1976), and it is not as common as pyruvyl or acetyl groups. The replacement of branched glucose by branched mannose residues in the repeating units is unusual. The distribution of these two repeating segments in the chain was not established, partly due to the lack of discrimination by the fungal cellulase. In principle, several possibilities exist. In the simplest case a distribution, according to unknown statistics, of the branched mannose and glucose residues within the same chain may occur (e.g. resembling the complex distribution pattern of guluronic acid and mannuronic acid residues in algal alginate). In contrast, a distribution of the two sugars in two sets of chains could be envisaged; however, intuitive biosynthetic considerations render such a hypothesis quite remote. The presence of two types of repeating units differing from each other for one sugar residue has been described, namely in the gellan-gum family of polysaccharides, where an α-L-mannopyranosyl residue partially replaces the α-L-rhamnopyranosyl residue either in the main chain [S-88 (Jansson et al., 1986) and S-198 (Chowdhury et al., 1987)] or as a single-unit side chain [S-130 (Jansson et al., 1985)]. The low molecular mass of the present EPS is a rather uncommon feature, since most known bacterial polysaccharides exhibit molecular masses of about 1 MDa.

A key step in the structural determination of this EPS was the use of a specific enzyme to obtain oligosaccharides corresponding to the repeating unit. The presence of the 4-*O*-L-Lac-β-D-Glc*p*A non-reducing terminus in the side chain suggested the use of bacteriophage φ22 endoglycanase (Parolis et al., 1988), since it was very effective in cleaving the *Klebsiella pneumoniae* K22 polysaccharide, which also possesses a tetrasaccharidic repeating unit with the same non-reducing terminus in the side chain. However, bacteriophage φ22 endoglycanase was totally ineffective on B62 EPS. Although the carboxylate residue is very important for the substrate specificity of this type of endoglycanases, it is probably not a sufficient structural condition, the other strict requirements being the position of the hydroxyl groups and the sterical arrangement (Geyer et al., 1983). On the contrary, the cellulase from *P. funiculosum* cleaved very efficiently the β(1−4) glycosidic linkages adjacent to the side chain. Moreover, the presence of mannose did not prevent the action of this enzyme. Another important feature of the cellulase is that the depolymerisation to the repeating units was complete only when B62 EPS had been de-*O*-acetylated previously. Treatment of the native polymer with cellulase led to the production of oligosaccharides consisting of multiples of the repeating unit, with and without *O*-acetyl groups. A hypothetical role for this substituent could be the protection of the EPS from the action of microbial endoglucanases. It has been reported that *Xanthomonas campestris* pv *campestris* produces endoglucanase (Gough et al., 1988), and this could be true also for *Xanthomonas campestris* pv *juglandis* (causal agent of walnut blight canker), although no such data are available. Several plant pathogenic bacteria produce endoglucanases, which are probably involved in the degradation of plant cell-wall glucans, thus facilitating the bacterial penetration into the plant tissues and /or help in the release of plant cell components that could be used as nutrients (Gough et al., 1988; Roberts et al., 1988; Boccara et al., 1994).

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REFERENCES

Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography, *Carbohydr. Res. 5*, 340−345.

Åman, P., Franzèn, L.-E., Darwill, J. E., McNeil, M., Darvill, A. G. & Albersheim, P. (1982) The structure of the acidic polysaccharide secreted by *Rhizobium phaseoli* strain 127 K38, *Carbohydr. Res. 103*, 77−100.

Amemura, A., Harada, T., Abe, M. & Higashi, S. (1983) Structural studies on the extracellular acidic polysaccharide from *Rhizobium trifolii* 4S, *Carbohydr. Res. 115*, 165−174.

Bax, A. & Summers, M. F. (1986) ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR, *J. Am. Chem. Soc. 108*, 2093−2094.

Boccara, M., Aymeric, J. & Camus, C. (1994) Role of endoglucanases in *Erwinia chrysanthemi* 3937 virulence on *Saintpaulia ionantha*, *J. Bacteriol. 176*, 1524−1526.

Bock, K., Lundt, I. & Pedersen, C. (1973) Assignment of anomeric structure to carbohydrates through geminal ^{13}C-H coupling constants, *Tetrahedron Lett. 13*, 1037−1040.

Bodenhausen, G. & Ruben, D. J. (1980) Natural abudance nitrogen-15 NMR by enhanced heteronuclear spectroscopy, *Chem. Phys. Lett. 69*, 185−189.

Chowdhury, T. A., Lindberg, B., Lindquist, U. & Baird, J. (1987) Structural studies of an extracellular polysaccharide (S-198) elaborated by *Alcaligenes* ATCC 31853, *Carbohydr. Res. 161*, 127−132.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. (1995) Microbial biofilms, *Annu. Rev. Microbiol. 49*, 711−745.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) Colorimetric method for determination of sugars and related substances, *Anal. Chem. 28*, 350−356.

Fett, W. F. (1993) Bacterial exopolysaccharides: their nature, regulation and role in host-pathogen interactions, *Curr. Top. Bot. Res. 1*, 367−390.

Fett, W. F., Osman, S. F., Wijey, C. & Singh, S. (1993) Exopolysaccharides of rRNA group I pseudomonads, in *Carbohydrates and carbohydrate polymers* (Yalpani, M., ed.) pp. 52−61, ATL Press, Inc., M Prospect IL.

Fett, W. F., Cescutti, P. & Wijey, C. (1996) Exopolysaccharides of th plant pathogens *Pseudomonas corrugata* and *Ps. flavescens* and th saprophyte *Ps. chlororaphis*, *J. Appl. Bacteriol. 81*, 181−187.

Gacesa, P. & Russell, N. J. (1990) Pseudomonas *infection and alginates Biochemistry, genetics and pathology*, Chapman and Hall, London.

Garozzo, D., Impallomeni, G., Spina, E., Sturiale, L., Cesàro, A. & Cescutti, P. (1995) Identification of *N*-acetylglucosamine and 4-*O* [1-carboxyethyl]mannose in the exopolysaccharide from *Cyanospir capsulata*, *Carbohydr. Res. 270*, 97−106.

Geyer, H., Himmelspach, K., Kwiatkowski, B., Schlecht, S. & Stirn S. (1983) Degradation of bacterial surface carbohydrates by viru associated enzymes, *Pure Appl. Chem. 55*, 637−653.

Gerwig, G. J., Kamerling, J. P. & Vliegenthart, J. F. G. (1978) Determination of the D and L configuration of neutral monosaccharides b high-resolution capillary G.L.C., *Carbohydr. Res. 62*, 349−357.

Gerwig, G. J., Kamerling, J. P. & Vliegenthart, J. F. G. (1979) Determination of the absolute configuration of monosaccharides in compl carbohydrates by capillary G.L.C., *Carbohydr. Res. 77*, 1−7.

Gough, C. L., Dow, J. M., Barber, C. E. & Daniels, M. J. (1988) Cloni of two endoglucanase genes of *Xanthomonas campestris* p *campestris*: analysis of the role of the major endoglucanase in path genesis, *Mol. Plant-Microbe Interact. 1*, 275−281.

Hakomori, S. (1964) A rapid permethylation of glycolipid, and pol saccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfo ide, *J. Biochem. (Tokyo) 55*, 205−208.

Hildebrand, D. C., Palleroni, N. J., Hendson, M., Toth, J. & Johnson, J. L. (1994) *Pseudomonas flavescens* sp. nov., isolated from walnut blight cankers, *Int. J. System. Microbiol. 44*, 410–415.

Hough, L. & Theobald, R. S. (1963) De-*O*-methylation with hydrobromic acid and boron tribromide, *Methods Carbohydr. Chem. 2*, 203–206.

Hurd, R. E. & John, B. K. (1991) Gradient-enhanced proton-detected heteronuclear multiple-quantum coherence spectroscopy, *J. Magn. Reson. 91*, 648–653.

Jansson, P.-E., Lindberg, B., Lönngren, J., Ortega, C. & Nimmich, W. (1984) Structural studies of the capsular polysaccharide of *Klebsiella* type 66, *Carbohydr. Res. 132*, 297–305.

Jansson, P.-E., Lindberg, B., Widmalm, G. & Sandford, P. A. (1985) Structural studies of an extracellular polysaccharide (S-130) elaborated by *Alcaligenes* ATCC 31555, *Carbohydr. Res. 139*, 217–223.

Jansson, P.-E., Kumar, N. S. & Lindberg, B. (1986) Structural studies of a polysaccharide (S-88) elaborated by *Pseudomonas* ATCC 31554, *Carbohydr. Res. 156*, 165–172.

Kenne, L. & Lindberg, B. (1983) Bacterial polysaccharides, in *The polysaccharides* (Aspinall, G. O., ed.) vol. 2, pp. 287–363, Academic Press, New York.

Kochetkov, N. K., Sviridov, A. F., Arifkhodzhaev, K. A., Chizhov, O. S. & Shashkov, A. S. (1979) The structure of the extracellular polysaccharide from *Mycobacterium lacticolum* strain 121, *Carbohydr. Res. 71*, 193–203.

Leigh, J. A. & Coplin, D. L. (1992) Exopolysaccharides in plant-bacterial interactions, *Annu. Rev. Microbiol. 46*, 307–346.

Lindberg, B., Lindqvist, B. & Lönngren, J. (1976) 4-*O*-[(*S*)-1-Carboxyethyl]-D-glucuronic acid: a component of the *Klebsiella* type 37 capsular polysaccharide, *Carbohydr. Res. 49*, 411–417.

Osman, S. F. & Fett, W. F. (1993) Structure of the acidic exopolysaccharide of *Pseudomonas marginalis* strain ATCC 10844, *Carbohydr. Res. 242*, 271–275.

Osman, S. F., Fett, W. F. & Dudley, R. L. (1994) Structure of the exopolysaccharide of *Pseudomonas stutzeri* strain ATCC 17588, *Carbohydr. Res. 265*, 319–322.

Palleroni, N. J. (1993) *Pseudomonas* classification: a new case history in the taxonomy of Gram-negative bacteria, *Antonie Leeuwenhoek 64*, 231–251.

Parolis, L. A., Parolis, H., Niemann, H. & Stirm, S. (1988) Primary structure of *Klebsiella* serotype K22 capsular polysaccharide: another glycan containing 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid, *Carbohydr. Res. 179*, 301–314.

Phillips, L. R. & Fraser, B. A. (1981) Methylation of carbohydrates with dimsyl potassium in dimethyl sulfoxide, *Carbohydr. Res. 90*, 149–152.

Pons, J. L., Malliavin, T. E. & Delsuc, M. A. (1996) Gifa V4: a complete package for the NMR data-set processing, *J. Biomol. NMR 8*, 445–452.

Prehm, P. (1980) Methylation of carbohydrates by methyl trifluoromethanesulfonate in trimethyl phosphate, *Carbohydr. Res. 78*, 372–374.

Roberts, D. P., Denny, T. P. & Schell, M. A. (1988) Cloning of the *egl* gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity, *J. Bacteriol. 170*, 1445–1451.

Sanford, P. A. & Baird, J. (1983) Industrial utilization of polysaccharides, in *The polysaccharides* (Aspinall, G. O., ed.) vol. 2, pp. 411–485, Academic Press, New York.

Sweet, D. P., Shapiro, R. H. & Albersheim, P. (1975) Quantitative analysis by various g.l.c. response-factor theories for partially methylated and partially ethylated alditol acetates, *Carbohydr. Res. 40*, 217–225.

Taylor, R. L. & Conrad, H. E. (1972) Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups, *Biochemistry 11*, 1383–1388.